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RESEARCH ARTICLE

Microbial analysis of cucumbers (*Cucumis sativus*) produced with tap or treated waste water

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Keywords
Coliforms; cucumbers; endophytic bacteria; irrigation water; microbial fruit invasion; treated waste water use; water contamination.

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Abstract

There is increasing evidence for a significant role of fruits and vegetables in infectious diseases in humans. Their consumption is increasing and environmental factors such as water availability are impacting their production. In this study, adding fertiliser to tap water (TW) increased the microbial load above that found in treated waste water (TWW); coliforms were also introduced. Low numbers of *Bacillus* spp. were recovered from inside some healthy cucumber fruits. No visible differences were observed between cucumber plants irrigated with TWW or TW or cucumbers with and without endophytic *Bacillus* spp. This is noteworthy when considering the use of TWW for crop irrigation.

Introduction

There is a consensus that diet can influence the risk of chronic diseases such as heart disease and cancer, and fruits and vegetables have been associated with reducing this risk (Van Duyn & Pivonka, 2000; Steffen, 2006). However, diet is also associated with acute infections and intoxications resulting from the consumption of contaminated foods. There is an increasing evidence that consumption of raw fresh fruits and vegetables is contributing to the continuously high incidence of human gastrointestinal illnesses (Sivapalasingam *et al*., 2004; Lynch *et al*., 2009). The consumption of these products has increased in many regions due to their promotion as 'healthy', including the '5-A Day for Better Health' program (Food Standards Agency (FSA), 2007).

Uncooked salad products are among the most frequently implicated fresh produce in outbreaks of disease, yet they are usually considered as low risk by consumers and as such are frequently subjected to minimal preparation (Little & Gillespie, 2008). The increased use and storage of preprepared salads augments the problem.

Raw and processed food produce are common exports in global markets and consequently, contamination at one source can result in geographically widespread problems (Little & Gillespie, 2008). The health, political and economic impact of these incidents was clearly demonstrated by the recent disease outbreak caused by shiga-toxin producing *Escherichia coli* on sprouts in Europe (Buchholz *et al*., 2011). Cucumbers were initially implicated resulting in large volumes being needlessly discarded and confusion among consumers as to who was to blame and what produce were safe.

The causative agents of food-borne illnesses are numerous and diverse and include bacteria such as Salmonella and pathogenic *E. coli*; contamination can occur at any point along the production route. Large-scale crop production requires irrigation. However, the frequent scarcity of good quality water in many regions of the world results in constraints on crop production (Ferrer *et al*., 2012). Irrigation is one of the major uses of water so better water management for this purpose could result in major improvements in water utilisation. Research is beginning to identify the complex pathways of transmission of water-borne pathogens and their interactions with produce. However, many gaps still exist in our knowledge and understanding. The health consequences of using waste waters for food crop irrigation are of considerable interest. Consequently the aim of this study was to analyse the microbiology of cucumbers

(*Cucumis sativus* L.), an important salad food, produced using either potable or treated waste water (TWW).

Materials and methods

Plant material and growing conditions

The experiment was carried out in a greenhouse environment during 2011 at the Agriculture Experiment Station facility (AES), Sultan Qaboos University, Oman. Greenhouse cucumber seeds were sown in nursery trays filled with growing substrate (Borgo, Terreau Universal Potting Substrate 50 L). Subsequently, 3-week-old uniform seedlings were transplanted into 35 cm plastic pots. The greenhouse temperature, $25 \pm 2^{\circ}$C and light, 240 μmol m^{-2} s^{-1} for a 14-h photoperiod, were maintained throughout the growing season. Pots were filled with equal amounts of potting media and vermiculite. The irrigation needs of the plants were monitored and 500 mL of the specified water type was applied on alternate days to meet the crop water requirement. Treated waste water was obtained from activated sludge processed at Sultan Qaboos University (SQU), Technical Affairs Department facility; both of the water samples used in this study were chlorinated. In the initial stages of plant growth (up to week 3), plants were supplemented with 250 mL of full strength Hoagland's nutrient solution once a week. As plant growth progressed this was increased to 500 mL (week 4) until final harvest (week 10). Volumes of the irrigation water were stored in plastic tubs in the greenhouse for use during the experiment. The water in the tubs was replenished every 2 weeks. The two types of irrigation water were stored under the same conditions, specifically $25 \pm 2^{\circ}$C, in non-light penetrating tubs with lids. Plant watering was carried out in a controlled fashion to avoid any direct contamination of the plant with the irrigation water. Plant soil pots were supplemented with full strength Hoagland's solution once a week (Hoagland & Arnon, 1950). Either tertiary TWW or TW was used for irrigation of the two independent 25 plant groups.

Plant growth parameters

Heights and yield of cucumbers per plant were recorded at the end of the experiment (week 12) using standard procedures.

Sample collection

Soil and irrigation water samples were collected aseptically into sterile bottles and transported to the laboratory on ice for immediate analysis. The fruit samples were collected from mature plants from week 5 to week 10 to estimate the microbial load. Five plants were randomly selected from each group and two mature fruits per plant harvested until four consecutive harvests were made (approximately 10 weeks). Harvesting was in the normal fashion but with minimal handling and fruits were packed into sterile bags and immediately transported to the laboratory for microbiological analysis (5–10 min from harvest to laboratory). Experimental randomised controlled design (RCD) was followed (Douglas, 2009).

Bacteriological analysis of soil and water

Three TWW samples, three TW samples (controls), one stock solution sample (Hoagland's) and one original soil sample were analysed prior to cultivating the plants. Subsequent samples of water were analysed at week 6 and week 10 (three replicates each time) and a repeat analysis of the plant soil growth medium was performed at the end of the experiment (week 12). Water and soil samples were analysed for coliforms, *E. coli* (ECC), and aerobic plate counts following standard procedures. Serial dilutions were made as required in maximum recovery medium (MRD). Estimation of aerobic plate counts was made using plate count agar (PCA). Total coliform and faecal coliform numbers were determined by simultaneously using violet red bile agar (VRBA) plates and 5 tube most probable number (MPN 5). VRBA plates were incubated at 35°C for total coliforms and at 44.5°C for faecal coliforms, both for 48 h. Inoculated lauryl tryptose broth tubes were incubated at 35°C for 48 h before examining for gas production. Tests were considered negative for coliforms if no gas was produced. Presumptive positive cultures were inoculated into brilliant green bile broth (BGBB) and incubated for 48 h at 35°C. Confirmed positive tubes with gas production were used to determine MPN for total coliforms. Positive cultures were transferred to fresh BGBB tubes and incubated at 44.5°C for 48 h. Positive tubes with gas production were used to determine MPN for faecal coliforms. All media were purchased from Oxoid, Basingstoke, UK.

Cucumber fruit examination

Freshly picked cucumber fruits were transferred directly to the laboratory and placed onto sterile surfaces. To prevent cross-contamination a number of agents were evaluated for their efficiency of decontaminating the surface of cucumbers before testing for microorganisms inside the fruit. These included ethanol (70% v/v) (J. T. Baker, MG Scientific, Pleasent Prairie, WI, USA), hydrogen peroxide (H_2O_2, 30% v/v) (Analar, BDH, Poole, UK) and UV light (lamp power approximately 125 μW cm^{-2} one metre from the lamp (Harrington & Valigosky, 2007), distance from lamp 50 cm, therefore approximately 500 μW cm^{-2})

for 20 min. Designated areas of the surfaces of the cucumbers were swabbed and inoculated onto PCA prior to any treatment. These areas were then washed with ethanol or H_2O_2 and the swabbing procedure was repeated. For UV decontamination a selected area on the cucumber surface was exposed to the UV source for 20 min, after which the area was again swabbed. Five cucumber fruits from independent plants were used for each treatment. Colony counts were determined after incubation at 37°C for 4 days. Microbial colonies were observed before all treatments and after cleaning with ethanol and H_2O_2 but no colonies were detected after exposure to UV light.

As the UV exposure was the most effective decontamination treatment this was subsequently used before recovering the internal content of cucumber fruits as outlined below. Cucumber fruits were placed individually on sterile plastic surfaces in a safety cabinet. An area of the surface was selected ($6 \times 2\,cm^2$), marked and directed towards the UV light source. The marked area was swabbed and aerobic plate counts performed. The cucumbers were then exposed to UV light for 20 min and the marked area recounted. To detect internal microorganisms the cucumber skin was peeled or cut away from the middle of the UV exposed area using a sterile knife. Sterile 1 mL syringes were used to puncture through the exposed internal area and 0.2 mL volumes of internal content were withdrawn from inside the cucumber and incubated on PCA (35°C, for 4 days), and VRBA (35°C and 44.5°C for 2 days).

Microbial identification

Bacterial isolates were identified using an automatic identification system (Vitek 2-compact15, bioMérieux, France) following the manufacturer's instructions. The Vitek 2 system and associated specialised cards are designed for identification of bacteria from diverse environments. Many studies have evaluated the Vitek 2 system and have reported high levels of reliability in identification to species level, comparable with other methods including molecular techniques; such studies include Halket et al. (2010) and Crowley et al. (2012).

Statistical analysis

Student's *t*-test was used for comparing the means of bacterial counts and the plant growth parameters.

Results

Plant growth parameters

No observable variations were detected in the growth patterns and fruit yields in response to the type of irrigation water. The mean heights of plant were 365.6 ± 10.8 and 369.8 ± 14.3 cm; and the cucumber yield per plant were 5.76 ± 0.41 and 5.86 ± 0.39 kg per plant [values quoted are means ± SD ($n = 5$), data not shown] for the TW and the treated water, respectively; analysis of the data identified no statistically significant differences between the height of plants ($P = 0.62$, *t*-test, $\alpha = 0.05$) or the fruit yield per plant ($P = 0.71$, *t*-test, $\alpha = 0.05$). The period between transplanting and the end of the experiment was 12 weeks.

Table 1 Initial microbial counts (colony forming unit, CFU) of irrigation waters before storage

	Mean counts ± SE			
	Aerobic plate	Coliforms		
Water sample	count (log CFU mL^{-1})	Plate (log CFU mL^{-1})	(MPN per 100 mL) Total	Faecal
Tap	ND	ND	0	0
Treated waste	3.01 ± 0.21	0.4 ± 0.4	5.3 ± 0.3	0

ND, no colonies detected ≤10 CFU mL^{-1}.

Microbial enumeration of irrigation waters

The aerobic plate counts of the fresh (immediately collected from the tap with no storage) TW were below the detection level (≤10 CFU mL^{-1}), while higher aerobic plate counts were obtained for the TWW [mean $1.1 \pm 0.42 \times 10^3$ ($n = 3$)]. No coliforms were isolated from fresh TW, in contrast, 100% of the fresh TWW samples contained coliforms, although these did not test positive as faecal coliforms. Plate detection of coliforms was less sensitive than the MPN-5 method with only 33% (one of three) of samples being positive when the plate method was used (Table 1).

Table 2 illustrates the counts of the water samples removed from the storage tubs during the experiment and the effect of adding fertiliser. The count of the waste water appeared to decrease during storage, in contrast to the counts of the TW which increased. Interestingly, no coliforms were isolated from water which had been stored. In all cases the count of the waste water exceeded that of the TW, but never statistically significant and the difference was considerably reduced when the fertiliser solution was added (Fig. 1).

Addition of fertiliser caused a significant increase in the total aerobic plate counts ($P = 0.02$, *t*-test, $\alpha = 0.05$) and also influenced the composition of the microflora including introducing coliforms. Indeed, addition of the fertiliser solution increased the microbial load and coliform content of the TW to above that of the fresh waste water samples (Table 2). The undiluted stock fertiliser solution

Table 2 Irrigation water microbial counts (colony forming unit, CFU) during the plant growth period

	log CFU mL^{-1} [a]			MPN index per 100 mL[a]	
Water sample	Total viable count	Total coliform	Faecal coliform	Total coliform	Faecal coliform
Stored tap	1.87 ± 0.17	ND	ND	<2	
Stored treated waste	2.75 ± 0.04	ND	ND	<2	
Tap + fertiliser	4.8 ± 0.04	ND	ND	191.5 ± 158.5	<2
Treated waste + fertiliser	5.23 ± 0	2.75 ± 0	2.44 ± 0.83	≥2400	<2

ND, no colonies detected ≤10 CFU mL^{-1}.
[a] Values are means ± SE.

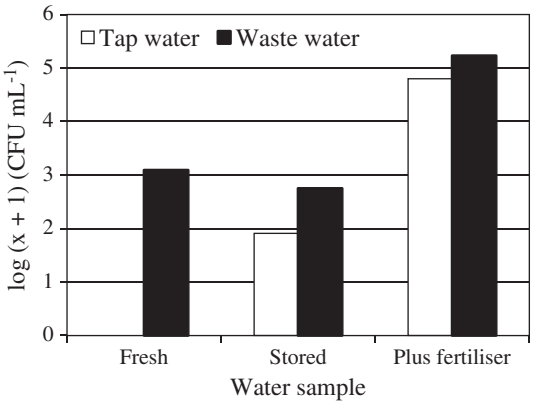

Figure 1 Change in water counts during storage and with the addition of fertiliser.

had a total plate count (PCA) of 9.1×10^4 CFU mL^{-1}; no *E. coli* or coliforms were confirmed directly from this. For the intense dark colour of the fertiliser solution making accurate counting and coliform detection difficult. However, coliforms were isolated from a diluted sample.

Microbial enumeration of soil samples

The microbial load and coliform content of the soils irrigated with waste water or TW were similar (Table 3). The total aerobic plate count (colony forming unit, CFU) was about 10^8 CFU g^{-1}, (slightly higher with TW) the coliform number was about 6×10^5 CFU mL^{-1}, (slightly higher for waste water) and the faecal coliform count was about 5×10^5 CFU mL^{-1} for soil from both groups.

Microbial enumeration of cucumber fruits

The mean aerobic plate counts (colony forming unit, CFU) on the surface of fruits from the two test groups were the same, approximately 1.4 CFU per 12 cm^2. The frequency of positive samples from inside the cucumbers was 35% for both irrigation water types, with no coliforms being isolated. The microbial counts inside the fruits irrigated with waste water ranged from 0 to

Table 3 Bacteriological counts (colony forming unit, CFU) of soil following irrigation with either tap water or treated waste water

	(Log CFU g^{-1})[a]		
Soil sample irrigation	Total count	Total coliform	Faecal coliform
Tap water	8.00 ± 0.58	5.23 ± 0.12	5.74 ± 0.03
Treated waste water	7.82 ± 0.23	6.00 ± 0.05	5.76 ± 0

[a] Values are means ± SE.

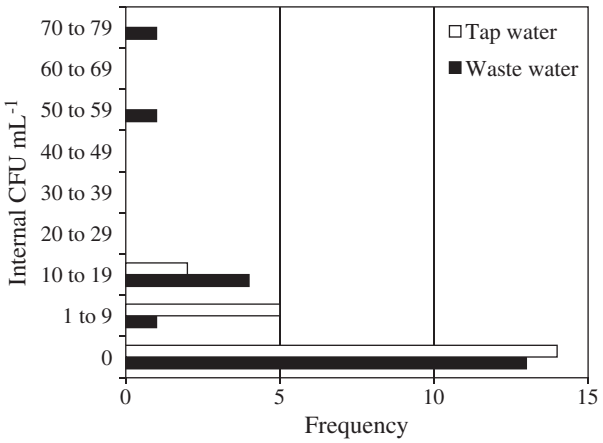

Figure 2 Frequency of microbial counts isolated from inside cucumbers (25 fruits for each irrigation type).

70 CFU mL^{-1} and with TW irrigation the range was 0–15 CFU mL^{-1}. The frequencies of aerobic plate counts are illustrated in Fig. 2. Using TWW for irrigation resulted in higher numbers of isolates being recovered from inside the cucumber but the numbers were not statistically significant ($P = 0.23$, t-test, $\alpha = 0.05$).

Identification of isolates

Table 4 shows the identification of isolates recovered during the experiment. All isolates from inside the cucumbers were identified with a high level of confidence (96–99%), as *Bacillus* ssp. for both water types. *E. coli* was isolated from soil that had been irrigated with stored TW.

Table 4 Identification and source of isolates

Cucumber inside Irrigated with		Stored water			Soil Irrigated with
Stored tap water	Stored waste water	Tap + fertiliser	Waste	Waste + fertiliser	stored tap water
Bacillus vallismortis (96%, excellent)	*Bacillus cereus*/*Bacillus thuringiensis*/*Bacillus mycoides* (slashline, very good)	*Acinetobacter lwoffii*/*Francisella tularensis* (low discrimination)	*Stenotrophomonas maltophilia* (99%, excellent)	*Enterobacter amino-genus*/*Ponatoea* spp./ *Leclerciaadecarboxylata* (low discrimination)	*Escherichia coli* (99%, excellent)
Bacillus subtilis/*Bacillus amyloliquefaciens*/*Bacillus atrophaeus* (slashline, excellent)	*Bacillus pumilus* (99%, excellent)	*Microbacterium* spp. (86%, acceptable)		*Klebsiella pneumoniae* spp. pneumoniae (98%, excellent)	
Bacillus pumilus (98%, excellent)					

The percentage in brackets is the level of confidence of the identification. Slash line indicates the accuracy of the genus identification and possible species. Low discrimination indicates possible identification.

Discussion

As expected the microbial load and coliform content of TWW was higher than that of TW. This did not have any observable effects on the development of the cucumber plants or fruits, and the counts for the soil samples for the two test groups were similar.

Addition of fertiliser to the irrigation water dramatically increased the microbial count and level of coliforms, indeed, raising the counts of the TW above those of the fresh waste water samples. Storing the water samples at 25°C, caused a slight reduction in the counts from the TWW while increasing those of the TW. Growth of bacteria including indicator organisms in water supplies is recognised and potential changes in the microbial load of water between its source and point of use are considered in water quality estimations (LeChevallier *et al*., 1996). There are a number of potential reasons for the slight increase in microbial count observed in stored TW. A number of studies have identified increases in the microbial levels of tap drinking water between the source and the point of use (Wright *et al*., 2004). During this study irrigation water was stored in tubs which had been cleaned but not sterilised. Coliforms were particularly sensitive to the storage conditions, being reduced to a level below the detection limit of plate counts. This observation could also support the theory that coliforms are subject to cell injury when suspended in water and that this contributes to the difference in sensitivity of the MPN-5 and plate count methods as injured cells have an impaired ability to grow on solid media. The number of coliforms detected in the stored TW was low, with a high level of variation. The nutrient content of the fertiliser may confer protection, enable damage repair, or potentially allow growth resulting in the increased number of coliforms detected.

Changes in the levels of chlorine and chlorinated compounds could also provide a potential explanation: during storage, volatiles will be lost which could lead to a reduction in the levels of agents which are inhibitory and/or injurious to bacteria (Levesque *et al*., 2006).

A variety of physical and chemical parameters including chlorine have been implicated in sublethal injury of coliforms in drinking water (McPeters *et al*., 1986). LeChevallier *et al*. (1996) reported that temperature also influences the microflora of drinking water; they demonstrated a positive correlation between temperature and number of coliform positive drinking water samples, up to 25°C. Increasing water temperature can increase the growth rate and yield of bacteria, with even small changes inducing significant changes in growth rate. *E. coli* was capable of only very slow growth in water at temperatures <20°C (Fransolet *et al*., 1985).

A range of potential human pathogens were isolated during the experiment. *Stenotrophomonas maltophilia* a ubiquitous, environmental bacterium frequently found on plants and soil was recovered from the TWW. It is an emerging, nosocomial, opportunistic pathogen which can cause severe infections particularly in the immunocompromised, often as a consequence of its enhanced ability to colonise surfaces (Ryan *et al*., 2009). Other workers have isolated *Stenotrophomonas* from untreated drinking water (Henriques *et al*., 2012). Although *S. maltophilia* poses potential health risks for humans it has been shown to develop beneficial interactions with plants including cucumbers. Isolates have been recovered from the surface of cucumber roots and from within the vascular structures of the root and stem (Mahaffee & Kloepper, 1997).

After addition of fertiliser to the TW, a species from the genus *Microbacterium* was isolated. *Microbacterium* spp. have been isolated from clinical specimens, although

their role in human disease remains unclear (Gneiding *et al*., 2008). Coliforms were identified from waste water supplemented with fertiliser; *Enterobacter* and *Klebsiella pneumoniae* are frequent isolates from water. Environmental isolates of *K. pneumoniae* have been shown to grow better than *E. coli* at low concentrations of organic carbon (Camper *et al*., 1991), which may contribute to their prevalence. *Leclercia adecarboxylata* which is naturaly found in the gut flora of animals, was also identified from waste water with fertiliser; it has many biochemical similarities to *E. coli* although its clinical significance is possibly limited. Most cases associated with *L. adecarboxylata* have been in immunocompromised patients, or have involved multiple causative agents (Hess *et al*., 2008). The only isolate confirmed as *E. coli* was recovered from soil irrigated with TW.

No isolates were recovered from inside the majority of the cucumbers. The number of colonies isolated from cucumbers irrigated with waste water was higher than when TW was used, although in both instances the counts were low. All isolates from inside the cucumbers were *Bacillus* spp. *Bacillus* spp. are endophytic and reports describe their isolation from a range of inner plant tissues. Endophytes generally are harmless to the plant, in fact, in most instances they stimulate the plant development and provide some protection against pathogenic invasion. It has been speculated that endophytes do not activate the defence responses as their numbers are low (Hallmann *et al*., 1997) and this is supported by this investigation where in most cases the number of isolates was in the range of 1–20 CFU.mL^{-1} and the plants and fruits did not demonstrate any signs of impairment. No attempt was made within this study to identify the source of entry of these organisms. Endophytes frequently enter plants through the root system, although aerial sites have been postulated. As care was taken with irrigation to prevent contamination of the plants it is most probable that entry was via the roots: endophytes can localise around the site of entry or disseminate through the plant (Zinniel *et al*., 2002).

Conclusions

This pilot study clearly demonstrates that microorganisms, specifically *Bacillus* spp. accumulate inside the cucumber fruit without visually detectable changes to the plant or the fruit. The microbial quality of the irrigation water can be compromised by the addition of fertiliser. Storage of irrigation water can lead to complex interactions between the microorganisms and the water environment which can result in cell growth, cell death or sublethal injury. A variety of potential human pathogens inhabit the cucumber growth environment and this will be the subject of future investigations, including their ability to enter healthy fruits.

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